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Immunocytochemical Studies on Lipid Droplet-Surface Proteins in Adrenal Cells

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Abstract Perilipin and ADRP, located on the surface of intracellular lipid droplets, are proposed to be involved in adipocyte lipid metabolism. The aim of the present study was to investigate the effect of PKA and PKC activities on the distribution of perilipin and ADRP in primary cultured adrenal cells, and the role of ERK in PMA- and calphostin C-induced steroidogenesis. Immunofluorescence staining indicated that in addition to p160, a capsular protein of steroidogenic lipid droplets, perilipin and ADRP were localized on the lipid droplet surface. Stimuli such as activation of PKA by db cAMP or inhibition of PKC by calphostin C, which increase corticosterone synthesis in various magnitudes, caused detachment of p160 and perilipin, but not ADRP, from the lipid droplet surface. Activation of PKC by PMA induced increase in corticosterone synthesis, however, it did not affect the distribution of perilipin, p160, or ADRP on the lipid droplet surface, suggesting the presence of mechanisms for promoting sterodiogensis other than causing detachment of lipid droplet surface proteins. We further demonstrated that ERK pathway was involved in PMA-induced steroidogenesis, since PD98059, specific inhibitor of MEK, blocked the increases in steroidogenesis and phosphorylation of ERK caused by PMA, but not by cAMP-PKA. These data indicate that p160, perilipin, and ADRP were all located on the lipid droplet surface in rat adrenal cells. On the basis of its non-responsiveness to lipolytic stimulation, ADRP may be a structural protein of the lipid droplet surface, whereas their immediate response to lipolytic stimuli suggest that perilipin and p160 are functional proteins. PKC regulates adrenal steroidogenesis through ERK cascade, whereas PKA pathway does not involve ERK. J. Cell. Biochem. 86: 432–439, 2002.

Key words: perilipin; adipose differentiation-related protein; p160; PKA; PKC; ERK; lipolysis

Perilipin and ADRP are well-characterized proteins of the lipid droplet surface in adipocytes [Greenberg et al., 1993; Londos et al., 1995; Brasaemle et al., 1997]. Perilipins A and B, isoforms produced by differential splicing of the same gene [Londos et al., 1995, 1996], can be phosphorylated by PKA, but remain on the lipid droplet surface during lipolytic stimulation

Abbreviations used: ADRP, adipose differentiation-related protein; CEH, cholesterol ester hydrolase; DMEM, Dulbecco's modified Eagle's medium; db cAMP, dibutyryl cyclic AMP; ERK, extracellular signal-related kinase; HSL, hormone sensitive lipase; MEK, mitogen-activated protein kinase kinase; PBS, phosphate-buffered saline; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol 12-myristate-13 acetate.

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[Londos et al., 1995, 1996]. Based on the fact that hormone-sensitive lipase (HSL) can be phosphorylated by PKA and translocated to the lipid droplet surface [Egan et al., 1992], Londos et al. [1995, 1996] proposed that perilipin may serve as a docking protein for HSL. Subsequently, Souza et al. [1998] reported that isoproterenol caused redistribution of perilipin from the lipid droplet surface to the cytoplasm, leading to the hypothesis that perilipin on the lipid droplet surface may prevent the binding of HSL to the lipid droplet, and thus prevent lipid hydrolysis by this enzyme. Moreover, expression of perilipin-cDNA increases triacylglycerol storage and decreases rate of lipolysis in 3T3-L1 preadipocytes [Brasaemle et al., 2001].

ADRP is highly expressed during preadipocyte differentiation [Jiang and Serreo, 1992; Ye and Serreo, 1998], and its mRNA is down-regulated in mature adipocytes, whereas the opposite is seen with perilipins [Brasaemle et al., 1997]. The finding that long-chain fatty acids induce COS-7 cells transfected with ADRP cDNA to accumulate lipid droplets in the cytoplasm supports for the role of ADRP in inducing the formation of lipid droplets [Gao and Serreo, 1999]. More evidence has been found in the etomoxir-treated rat, in which induced ADRP expression and lipid accumulation are seen in hepatocytes [Steiner et al., 1996], and in foam cells in artherosclerotic lesions, in which correlative ADRP gene expression and lipid accumulation are seen [Wang et al., 1999].

Using a specific monoclonal antibody, known as A2, our laboratory has identified a capsular protein, p160, which is found on the surface of steroidogenic lipid droplets in rat adrenal cells and is detached by stimuli, such as PKA activation or PKC inhibition [Wang and Fong, 1995; Fong and Wang, 1997], treatments increasing steroidogenesis in these cells. P160 may, therefore, act as a protein barrier on the lipid droplet surface, regulating the process of lipolysis. It was of interest to know whether another two lipid droplet surface proteins, perilipin and ADRP, also respond to PKA activation and PKC inhibition. In addition, data on the rates of detachment from the lipid droplet surface of these three proteins after stimulation may provide new insights into the stability of these proteins.

Recent studies indicate the possible involvement of ERK in lipolysis. Phosphorylation of CEH by gonadotropin in granulose cell lines decreased the expression of steroidogenic acute regulatory protein and steroidogenesis [Seger et al., 2001], whereas phosphorylation of ERK by dioctanoglycerol in 3T3-adipocytes [Greenberg et al., 2001] or by angiotension II in bovine glomerulosa cells enhanced HSL activity and promoted lipolysis [Cherradi et al., 2001; Greenberg et al., 2001]. However, the role of ERK in steroidogenesis in rat adrenal cells remains to be elucidated.

Most studies on perilipin and ADRP have used cell lines, including 3T3-L1 preadipocytes, MA-10 Leydig cells, and adrenal tumor Y-1 cells, as experimental models. However, phenomena observed in cell lines do not necessarily occur in situ. In order to solve the questions raised above, we designed experiments using primary adrenal cell cultures to study (1) the distribution patterns of ADRP and perilipin, with particular focus on their location relative to p160, (2) the effects of PKA and PKC on the distribution of ADRP and perilipin on the lipid droplets, and (3) the possible involvement of ERK in PMA-stimulated steroidogenesis.

MATERIALS AND METHODS

Cell Culture

Adult female Wistar rats (8–12 weeks of age) were used. The animals were killed by intraperitoneal injection of 7% chloral hydrate (6 ml/kg), and primary cultures of adrenocortical cells were prepared as described previously [Wang and Fong, 1995]. Briefly, adrenal gland fragments were incubated in serum-free DMEM (GIBCO, Grand Island, NY) containing 1 mg/ml of type II collagenase (C-6885, Sigma, St. Louis, MO) for 15 min in a 37°C water bath, then the reaction was stopped by addition of ice-cold culture medium (1:1 v/v mixture of Ham's F12 medium and DMEM, supplemented with 25 mM HEPES, 1.2 g/L of $NaHCO_3$, 5% horse serum, 2.5% fetal bovine serum, and 1% penicillin and streptomycin). The dissociated cells were collected by centrifugation at 1,000*g* for 10 min, resuspended, and seeded on coverslips (for immunostaining) or in 24-well plates (for radioimmunoassay) and maintained for 3 days at 37°C in a 95% air/5% CO_2 atmosphere.

Western Blot Analysis

Adrenal cultures were determined using the BioRad protein assay kit. Samples (80 μg of

protein/lane) were electrophoresed on a 10% sodium dodecyl sulfate–polyacrylamide gel and the proteins transferred to nitrocellulose paper as described by Fritz et al. [1989]. Membrane strips were blocked for 1 h at room temperature with 5% non-fat milk in Tris-buffered saline (150 mM NaCl, 50 mM Tris-base, pH 8.2), then incubated overnight at 4°C with primary rabbit anti-perilipin [Souza et al., 1998], anti-ADRP antibodies (a kind gift from Dr. Thomas Keenan; Heid et al., 1996], or anti-phosphorylated ERK (Santa Cruz, CA) diluted in PBS. After washes with Tris-buffered saline-0.1% Tween, the strips were reacted with alkaline phosphatase-conjugated second antibodies (1:7,500 dilution, Sigma) and bound antibody visualized using a substrate solution (3.3 mg/ml nitro blue tetrazolium and 1.65 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in 100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-base, pH 9.5)

EXPERIMENTS

Drug Treatments

The PKC activator, phorbol-12-myristate-13-acetate (PMA; Calbiochem, San Diego, CA), the PKC inhibitor, calphostin C, and the cAMP analogue, dibutyryl cAMP (db cAMP, Sigma) were used to manipulate the activities of PKC and PKA. Adrenal cells were treated with 1 mM db cAMP, 500 nM calphostin C, or 100 nM PMA for 1–3 h prior to immunofluorescence analysis. The ERK inhibitor, PD98059, was purchased from Calbiochem and used at the working concentration of 40 μM. All experiments were performed in triplicate and more than 100 cells were examined. The percentages of stained cells were expressed as the means ± SD.

Immunofluorescence

Cultured adrenocortical cells were fixed for 5 min at room temperature in 0.15% glutaraldehyde in PBS (pH 7.4) and permeabilized for 10 min at room temperature with 0.15% Triton X-100 in PBS. To reduce free aldehyde groups, the cells were then treated for 30 min at room temperature with $NaBH_4$ (1 mg/ml). After washes in PBS, the cells were single-labeled for 2 h at 37°C with mouse monoclonal antibody A2 (IgM) (1:200 dilution of ascites in PBS), which labels the lipid droplet surface capsule [Wang and Fong, 1995], with rabbit anti-ADRP or with rabbit anti-perilipin antibodies. Cells were also double-labeled for p160 and ADRP or perilipin. They were then washed with PBS (3 × 5 min) and reacted with FITC-conjugated goat anti-mouse IgG or anti-rabbit IgG (Sigma) for single labeling or with a mixture of Texas Red-conjugated goat anti-mouse IgM antibodies (μ-chain specific; Vector, Burlingame, CA) and FITC-conjugated goat anti-rabbit IgG antibodies (γ-chain specific; F-8264, Sigma) for double-labeling. After incubation for 1 h at 37°C, the cells were washed extensively with PBS, mounted using 3% n-propyl gallate and 50% glycerol in PBS, and examined using a Zeiss fluorescence microscope.

Corticosterone Radioimmunoassay

After drug treatment, 5 μl of the culture medium was diluted (1:100) with the assay buffer (0.05 M Tris-HCl, pH 8.0, containing 0.1 M NaCl, 0.1% NaN_3, and 0.1% of bovine serum albumin) and incubated with diluted rabbit anti-corticosterone (Sigma). After 20 min at 37°C, 100 μl of ^{3}H-corticosterone (10,000 cpm in assay buffer) was added to the mixture and incubated for 1 h at 37°C and then 1 h at 4°C. The free hormones were adsorbed on 200 μl of dextran-coated charcoal (0.5% dextran and 1.25% charcoal in assay buffer for 10 min and the bound hormones separated by centrifugation at 13,000 rpm for 10 min. The supernatant (about 0.7 ml) was transferred to a counting vial containing 3 ml of counting solution (Ecoscient H) before counting in a β-counter (LS600IC, Beckman, Fullerton, CA). A standard curve was established using corticosterone standard (Sigma).

Statistical Analysis

All results are expressed as the mean ± SD. Statistical differences between means were assessed using Student's *t*-test, a *P* value of less than 0.05 was considered significant.

RESULTS

Antibody Specificity in Adrenal Cells

Western blot analysis of purified lipid droplets showed that the anti-ADRP antibody and anti-perilipin antibody recognized, respectively, 50 kDa ADRP (Fig. 1, lane B) and 58 kDa perilipin A (Fig. 1, lane C), while controls lacking primary antibody showed no background reaction (Fig. 1, lane A). Antibody A2 reacted with a 160 kDa capsular protein (P160)

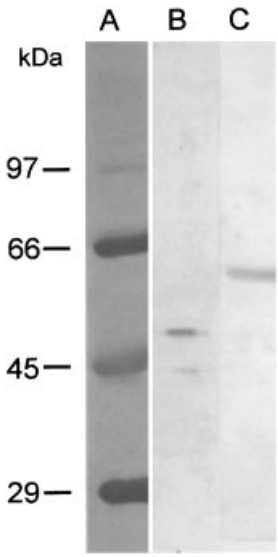

Fig. 1. Western blot analysis of lipid droplets isolated from adrenal cortex using rabbit polyclonal anti-ADRP and anti-perilipin antibodies. (**A**) Molecular weight standards. (**B**) Anti-ADRP, showing the 50-kDa ADRP band. (**C**) Anti-perilipin antibody, showing the 58-kDa perilipin band.

(data not shown), as previously reported by Wang and Fong [1995].

Distribution of p160 and Perilipin in Adrenal Cells

Immunofluorescence studies showed perilipin and p160 to be distributed on the lipid droplet surface in the form of rings (Fig. 2A,B), although not all cells were simultaneously positive for both. For convenience of description, we classified double-labeled cells into four staining patterns, A^+P^+ (positive for both p160 and perilipin; Fig. 2A,B), A^+P^- (positive only for p160: Fig. 2C,D), A^-P^+ (positive only for perilipin; not shown), and A^-P^- (negative for both; Fig. 2E,F). In normal control cells, A^+P^+, A^+P^-, and A^-P^- cells constituted 71.4, 10.2, and 18.3%, respectively, of the total cell population, while A^-P^+ cells accounted for less than 5%.

Effects of PKA and PKC on Corticosterone Production

Treatment with db cAMP, PMA, or calphostin C increased corticosterone production to 12.4-, 3.2-, or 1.7-fold of controls, respectively (Fig. 3). Therefore, activation of PKA, and activation or inhibition of PKC all increased steroidogenesis, though in different degrees.

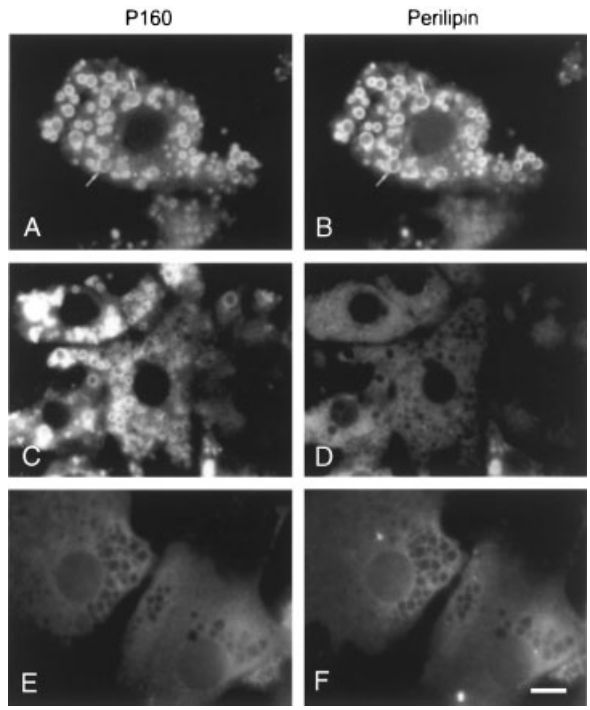

Fig. 2. Double immunofluorescence labeling for perilipin and p160 in normal adrenal cells. Cells were double-stained for p160 (**A**, **C**, and **E**) and perilipin (**B**, **D**, and **F**). Most show positive staining for p160 and perilipin (A^+ P^+) on the lipid droplet surface (arrows in A and B). Others are only positive for p160 (A^+ P^-) (C and D) or negative for both (A^-P^-) (E and F). Scale bar, 10 μm.

Effects of PKA Activation on the Distribution of p160 and Perilipin

Following PKA activation by db cAMP, perilipin staining on the lipid droplet surface became weak after 1 h of db cAMP treatment (Fig. 4B compared to control in Fig. 2B) and was

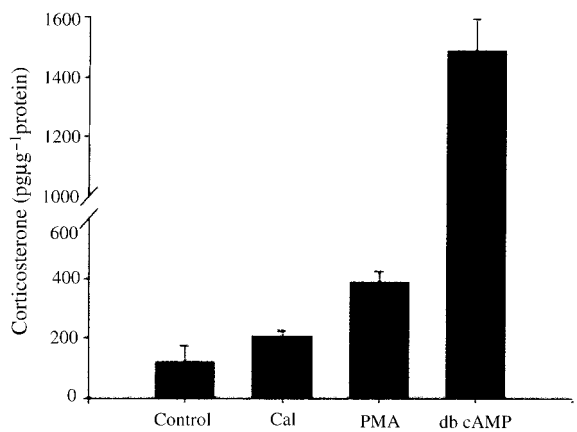

Fig. 3. Effects of db cAMP, PMA, and calphostin C on corticosterone production. Values are mean ± SD; n = 3.

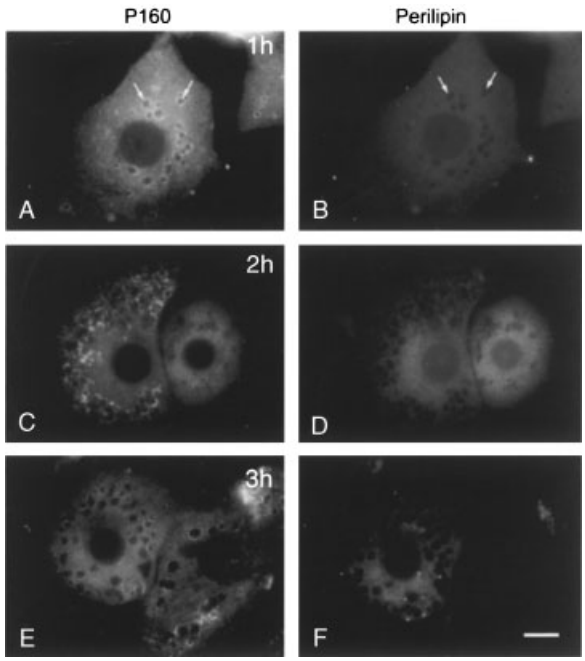

Fig. 4. Effect of db cAMP on the distribution of p160 and perilipin. Cells were treated with 1 mM db cAMP for 1 h (**A** and **B**), 2 h (**C** and **D**), or 3 h (**E** and **F**), and double-stained for p160 (A, C, and E) and perilipin (B, D, and F). Perilipin staining was weak 1 h after treatment (arrows in B), while p160 staining remained strong (arrows in A). P160 and perilipin were not detected on the surface of lipid droplets after 3 h of treatment (E and F). Scale bar, 10 μm.

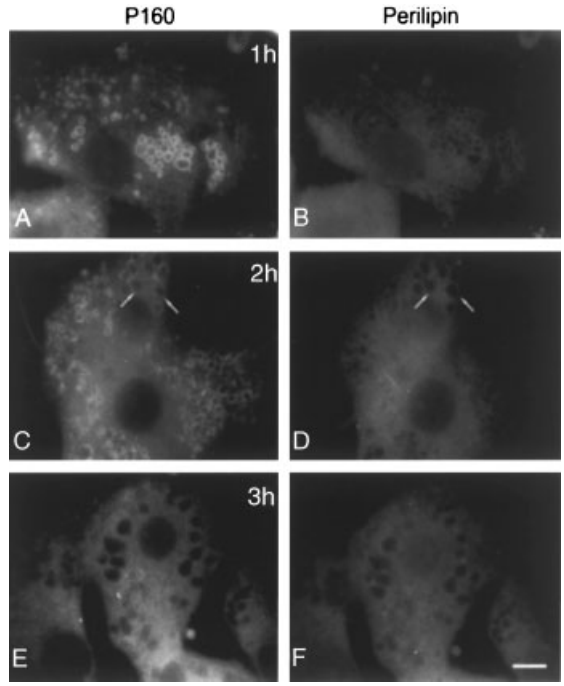

Fig. 5. Effect of calphostin C on the distribution of p160 and perilipin. Cells were treated with 500 nM calphostin C for 1 h (**A** and **B**), 2 h (**C** and **D**), and 3 h (**E** and **F**), then double-stained for p160 (A, C, and E) and perilipin (B, D, and F). After 2 h of treatment, P160 staining was discontinuously distributed on the lipid droplet surface (arrows in C) and perilipin staining was undetectable on the surface of lipid droplets (arrows in D). Staining for p160 and perilipin was not seen on lipid droplets after 3 h of treatment (E and F). Scale bar, 10 μm.

lost after 2 h of treatment (Fig. 4D). P160 staining was unchanged after 1 h (Fig. 4A), but began to form a discontinuous ring pattern after 2 h of treatment (Fig. 4C). After 3 h of treatment, neither protein was detectable on the lipid droplet surface (Fig. 4E,F). The proportion of A^+P^+ cells decreased to 50.2, 48.4, or 8.9%, respectively, after 1, 2, or 3 h of treatment, while the proportion of A^-P^- cells increased.

Effects of PKC Activity on the Distribution of p160 and Perilipin

The distribution of p160 (Fig. 5A) and perilipin (Fig. 5B) was unaffected after 1 h of treatment with calphostin C (PKC inhibitor). After 2 h of treatment, loss of perilipin staining (Fig. 5D) and discontinuous p160 staining on lipid droplet surface (Fig. 5C) were noted, while after 3 h, neither protein was detectable on the lipid droplet surface (Fig. 5E,F). These morphological observations correlated well with the quantitative analysis, which showed that the proportion of A^+P^+ cells decreased to 38, 31, or 13.9%, while that of A^-P^- cells increased to 43, 55, or 79%, after, respectively, treatment for 1, 2, or 3 h.

After PKC activation by PMA, most perilipin and p160 staining was still localized on the lipid droplet surface in 65% of the cells, not significantly different from the results in control cells (data not shown).

Effects of PKA and PKC Activity on the Distribution of p160 and ADRP

Figure 6 shows colocalization of p160 (Fig. 6A) and ADRP (Fig. 6B) on the lipid droplet surface in the majority of adrenal cells. In the control group, 90% of cells were immunostained for ADRP. Activation of PKA by db cAMP (Fig. 6D), activation of PKC by PMA (data not shown), or inhibition of PKC by calphostin C (Fig. 6F) had no effect on the distribution of ADRP on the lipid droplet surface, whereas all of these treatments resulted in p160 being lost (Fig. 6C,E).

Involvement of ERK in PMA-Induced Steroidogenesis

It has been shown that catecholamines stimulate ERK cascade, which phosphorylates

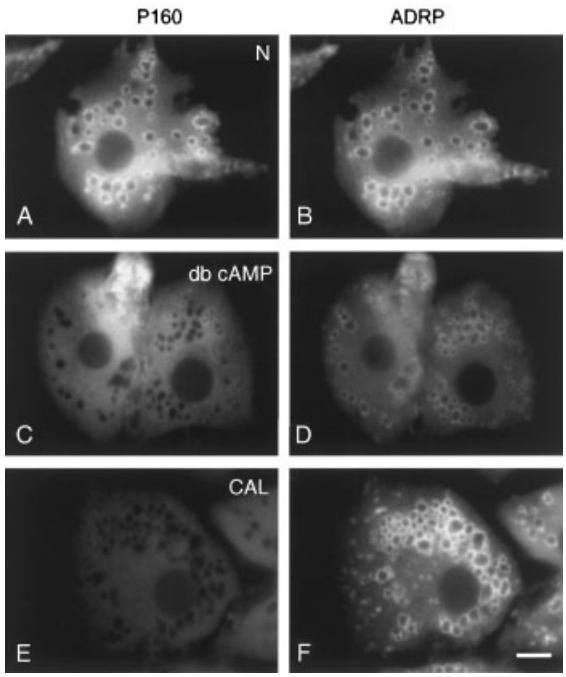

Fig. 6. Effects of db cAMP and calphostin C on the distribution of p160 and ADRP. Cells were untreated (**A** and **B**) or treated with 1 mM db cAMP (**C** and **D**) or 500 nM calphostin C (**E** and **F**) for 3 h, followed by double-labeling for p160 (A, C, and E) and ADRP (B, D, and F). ADRP staining was not affected by db cAMP and calphostin C (D and F), while p160 staining on lipid droplet surface was lost (C and E). Scale bar, 10 μm.

HSL and increases lipolysis, in 3T3-L1 adipocytes [Greenberg et al., 2001]. In the present study, co-treatment of PMA with PD 98059 significantly blocked steroidogenesis induced by PMA, but not by db cAMP (Fig. 7). Phosphorylation of ERK1 and ERK2 was detected after treatments with PMA for 30 min (Fig. 8C), and the PMA-induced phosphorylation of ERK1 and ERK2 was inhibited by PD 98059 (Fig. 8D).

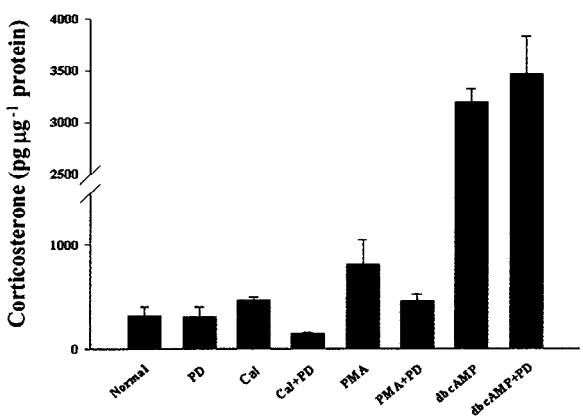

Fig. 7. Effects of PD98059 on db cAMP- and PMA-mediated steroidogenesis. Adrenal cells were treated with PD98059 (40 μM) alone or in combination with db cAMP (1 mM), PMA (100 nM), or calphostin C (500 nM) for 6 h. The culture supernatants were assayed for corticosterone production. Values are mean ± SD; n = 3.

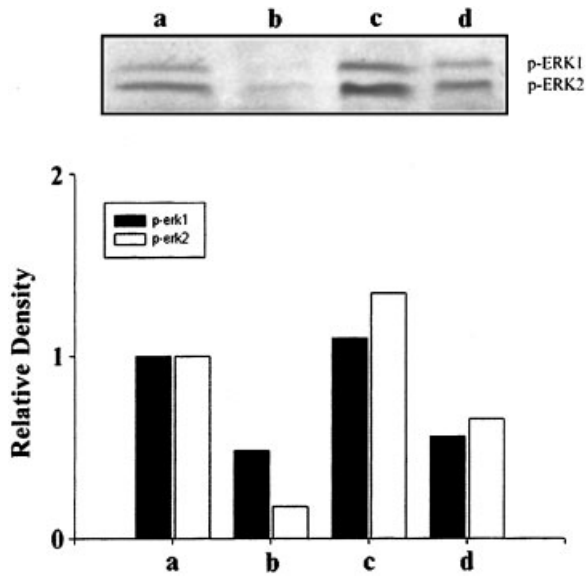

Fig. 8. Phosphorylation of ERK by PMA treatment. Upper trace: Immunoblots with anti-phosphorylated ERK antibody. **Lane a**: Control cells. **Lane b**: PD98059-treated cells. **Lane c**: PMA-treated cells. **Lane d**: PD98059 plus PMA-treated cells. Lower trace: Densitometric scans of the same blots, representing a typical example from three independent experiments.

DISCUSSION

Lipid droplets isolated from rat adrenal cells contain at least 10 different proteins by gel analysis (our unpublished data). The present study confirmed that in addition to p160, ADRP and perilipin are also localized on the lipid droplet surface of rat adrenal cells. The observation that both perilipin and ADRP were present in mature, large lipid droplets in rat adrenal cells differs from results in 3T3-L1 adipocytes, in which ADRP is only expressed during the early differentiation stage and not expressed in mature adipocytes, whereas perilipin is consistently expressed in mature adipocytes [Brasaemle et al., 1997]. A similar coexistence of ADRP and perilipin is seen in lipid droplets in adrenal Y-1 cells and MA-10 Leydig cells [Jiang and Serreo, 1992; Servetnick et al., 1995; Brasaemle et al., 1997a].

Coexistence of perilipin and p160 at the lipid droplet surface was seen in 70% of control adrenal cells, but not in 30%, suggesting that the latter population might be actively involved

in basal rate of corticosterone synthesis. In this study, perilipin became detached from the lipid droplet surface before p160 in response to db cAMP and calphostin C. This may be due to the fact that perilipin has multiple phosphorylation sites that can be responsive to drug stimulation. Another explanation is that the binding affinity between perilipin and the outer phospholipid layer of the lipid droplet is weaker than that of the other two lipid droplet surface proteins (p160 and ADRP). The data suggest that perilipin and 160 kDa protein are much more unstable lipid droplet surface proteins than ADRP, which was remarkably resistant to treatment with db cAMP, PMA, or calphostin C. Our results are consistent with the hypothesis that perilipin acts as a barrier for the access of HSL, as proposed by other investigators [Barber et al., 1995; Souza et al., 1998]. Lipolysis of isolated lipid droplets could be induced by okadaic acid in the presence of HSL, while artificial lipid droplets (trioleoylglycerol emulsified with gum arabic) were not responsive, suggesting lipid droplet-associated proteins may also mediate the binding of HSL to the droplet surface [Morimoto et al., 2000].

It is noteworthy that similar proportions of A^-P^- cells were seen in the db cAMP-treated and calphostin C-treated groups, despite the fact that the magnitude of db cAMP-induced steroidogenesis was far greater than that caused by calphostin C. It is apparent that the detachment of lipid droplet surface proteins is not the only reason for increased lipolysis, since decapsulation of lipid droplets simply provides better access of active HSL or CEH to the stored triacylglycerol or cholesterol ester in lipid droplets, and the activities of HSL or CEH evoked by different stimuli should also be considered. Activation of PKA by db AMP phosphorylates HSL and enhances lipolysis in 3T3-adipocytes [Egan et al., 1992] or phosphorylates CEH and increases corticosterone production in adrenocortical cells [Beckett and Boyd, 1977]. Therefore, even the proportions of A^-P^- cells in db cAMP-treated and calphostin C-treated groups were similar, the activities of HSL may be higher in the former group, which may be responsible for the higher steroid production efficiency.

In the present case of PMA-stimulated rat adrenal cells, phosphorylation and activation of ERK was apparently related to steroid production. As to how PMA increased phosphorylation of ERK, it may be operated as reported in 3T3 fibroblasts that activated PKC can phosphorylate and activate c-Raf, leading to stimulation of the ERK cascade [Kolch et al., 1993; Marais et al., 1998]. ERK1 and ERK2 are found to mediate forskolin-induced steroid secretion in Y1 mouse adrenocortical cells [Gyles et al., 2000]. It has also been reported that ERK activation phosphorylates HSL and increases lipolysis in 3T3-adipocytes [Greenberg et al., 2001]. Thus, PKC might indirectly activate CEH via ERK or directly activate CEH as in Y1 mouse adrenocortical cells [Balkow et al., 1990]. In the present study, PKA system activated by db cAMP did not phosphorylate ERK, and inhibitor of MEK did not prevent db cAMP-induced steroidogenesis. Therefore, in rat adrenocortical cells, PKA activated by db cAMP failed to induce ERK activation (data not shown), unlike the cases in rLHR-4 and rFSH-17 granulosa-derived cell lines [Seger et al., 2001]. Similar to the finding that PKA and PKC regulate steroidogenesis through independent signaling pathways and exert different effects on steroid hydroxylase gene expression in Y1 mouse adrenocortical cells [Reyland, 1993], our data also show that PKA and PKC affect steroidogenesis and protein distribution on lipid droplet surface by independent signaling pathways.

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